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Terminal Long Tandem Repeats in Chromosomes from *Chironomus pallidivittatus*

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We provide evidence that a chromosome end in the dipteran *Chironomus pallidivittatus* contains 340-bp tandem repeats reaching the extreme terminus of the chromosome. After adding synthetic oligonucleotide tails to DNA extracted from the microdissected right end of the fourth chromosome, we could demonstrate that the blocks of repeats were tailed at only one end, the chromosome terminus, the interior of the arrays being unavailable for tailing. Using PCR, we furthermore showed that the added tails were connected to 340-bp repeat DNA directly, i.e., without intervening DNA of any other kind. The tailed repeats belong to a subfamily previously known to be the most peripheral one of the different types of 340-bp units. Using plasmid controls, we could also make certain that we did not amplify rare or nonrepresentative DNA termini.

Telomeres of most investigated eukaryotic species terminate with short repeats, usually with guanine excess in the 3′-terminating strand (4). Such repeats, whether regular, as in most cases (5, 17, 21, 25), or irregular, as in *Saccharomyces cerevisiae* (29) among others, are likely to result from telomerase activities (6, 11, 16, 31).

Short repeats are also found in several insect orders (23), but for the dipteran *Drosophila melanogaster* a different mode of chromosome termination has been established. Here terminally deleted chromosomes can be capped by non-long-terminal-repeat retrotransposons (2, 3, 30, 33), which can also be recovered at the ends of normal chromosomes (15).

No other type of ending is known for nuclear chromosomes, but the linear mitochondrial DNA from *Tetrahymena thermophila* ends with >50-bp-long repeat units (19, 20). We present evidence that this alternative, here in the form of 340-bp tandem repeats (27), is used by nuclear chromosomes in the dipteran *Chironomus pallidivittatus.*

The 340-bp tandem repeat family is localized at seven of the eight (pairs of) chromosome ends in <200-kb blocks. Although the blocks are close to the chromosome ends as revealed by *Bal* 31 digestions (34), it has not been known whether they reach the extreme termini. The repeat family contains four different subfamilies, three of which, D1, D2, and D3, are derived from the fourth one, M1, each by a cluster of mutations in short regions designated ds1, ds2, and ds3, respectively (7). In the case of D3 there is a second difference, i.e., one large region has become substituted for another one. Telomeres differ in subfamily composition (8, 34). When D3 is present together with other kinds of repeats, it is always the most peripheral unit (34).

Briefly, our strategy was to gently release DNA from single microdissected right ends of the fourth chromosome and provide it with tails. Anchors added to the tails and primers for the 340-bp repeat were used for PCR amplifications. We could demonstrate that no internal breaks were tailed within the repeat blocks and that the tails were added directly to the 340-bp repeat DNA. We also showed that only DNA from a specific subfamily, previously shown to be distal (34), was amplified, although the genomic primer was hybridizing to all subfamilies with the same efficiency. Using plasmid controls, we could also make certain that we have not amplified rare or nonrepresentative DNA termini. The G-rich strand in these repeats (22) is oriented with its 3′ end towards the terminus as in short telomeric repeats. Since all seven chromosome ends contain similar blocks of 340-bp repeats, it is likely that their telomeres all have similar terminations.

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MATERIALS AND METHODS

DNA isolation. We used microdissected material to identify the chromosomal origin of the DNA and decrease its complexity. Salivary glands from late-fourth-instar larvae of *C. pallidivittatus* were fixed in 70% ethanol for 30 min, transferred to ethanol-glycerol (1:1), and arranged in an oil chamber (24). Nuclei were removed, and polytene chromosomes were individually released. All ends were identified and cut off, releasing fragments encompassing several hundred kilobases more DNA than the <200-kb blocks of 340-bp repeats. Up to about 10 pieces from one kind of chromosome end, e.g., 4R, constituted one sample, and such samples were produced from the different dissected ends, after which DNA was extracted as follows: in the oil chamber the samples were separately picked up on the tip of a glass needle and transferred to an 0.5-ml Eppendorf tube containing 50 μl of a mixture of 0.5 mg of proteinase K per ml, 10 mM Tris-Cl (pH 8.0), 10 mM NaCl, and 0.1% sodium dodecyl sulfate, where it was digested at 37°C for 60 min and gently treated with phenol, phenol-chloroform, and chloroform. The chloroform was removed with diethylether, which in turn was eliminated by evaporation.

PCR amplification of chromosome ends. DNA extracted from the chromosome ends was heated at 55°C for 5 min, cooled, and then incubated with 25 U of terminal deoxynucleotide transferase (Boehringer) in 60 μl of a mixture of 0.2 M potassium cacodylate, 25 mM Tris-Cl (pH 6.6), 0.25 mg of bovine serum albumin per ml, 0.75 mM cobalt chloride, 750 μM dCTP, and 37.5 μM ddCTP for 90 min at 37°C. The enzyme was inactivated at 75°C for 10 min, and the tailed DNA was then lightly sheared to facilitate its dissolution after ethanol precipitation.

PCR with an anchored primer was then performed essentially according to the rapid amplification of cDNA ends technique (10). Two-fifths of the tailed DNA were first used as a template for asymmetric PCR in 50 μl with 0.1 μM anchorage R_0-R_1-$(dG)_{14}$ primer (Table 1) in a mixture of 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (dNTPs), 0.25 U of Amplitaq (Perkin-Elmer), 0.001% (wt/vol) gelatin, 63 mM KCl, and 12.5 mM Tris-Cl buffer, pH 8.3. Hot-start PCR (Ampliwax; Perkin-Elmer) with annealing at 59°C was used with 10 cycles of 1-min 94°C denaturation, 2-min 59°C annealing, and 2-min 72°C extension. The same procedure was applied to the recombinant plasmid used as a control, pET22, which contains 10 copies of 340-bp repeats inserted between the *Sma*I and *Acc*I sites of pUC18. Before tailing, the plasmid had been cut with either *Pst*I or *Kpn*I. The resulting 3′ protrusions were tailed after ethanol precipitation, and the constructs were quantitated spectrophotometrically. The sizes of the bands expected from amplification with L3C and L3W primers are given in Table 1.

For amplification of the asymmetric reaction volumes, we used 10 μl as a template in final volumes of 50 μl. R_0 primer (0.2 μM) and either L3C or L3W primer (0.4 μM) (Table 1) were used in a touchdown initiation procedure in which the start was at 67°C and each subsequent cycle was at a 1°C-lower temperature during 7 cycles, after which 60°C was maintained for another 30 cycles (1-min 94°C denaturation, 1-min annealing, and 1-min 72°C extension,

TABLE 1. Oligonucleotides used to characterize the termini of *C. pallidivittatus* telomeres and to quantitate subfamily contents

Primer	Sequence	Expected size(s) of amplified bands (bp) in control plasmid
$R_0R_1G_{14}$	GCTAAGTCGGGAGCTCTACAAGCAAGGATCCGTCGACATCG$_{14}$	
R_0	GCTAAGTCGGGAGCTCTACAAGC	
R_1	AAGGATCCGTCGACATC	
L3C	TTAGGTGATGAGGGGTCGTGCAT	412, 751, 1,090...
L3W	ATGCACGACCCCTCATCACCTAA	138, 477, 816...
L1-IIA-C	GGAGCTAGGACGATTTTTCCAAT	

with a final extension for 10 min at 72°C). One-fifth of the reaction mixtures was electrophoresed without further treatment in 0.8% agarose gel, transferred to a nylon membrane, and hybridized with radiolabelled 340-bp repeat. Another fifth of the reaction mixtures was digested in 200 μl with 8 U of S1 nuclease at 37°C for 30 min, precipitated after inactivation with EDTA, and then electrophoresed.

Cloning and sequencing of amplified chromosome ends. For sequencing, 1% of the nuclease S1 reaction volume was first reamplified in 50 μl of a mixture containing 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.25 U of Amplitaq, 10% glycerol, 63 mM KCl, and 75 mM Tris-Cl buffer, pH 8.7. The primers were L1-IIA-C, complementary to the border region between linker region L1 and the subrepeat IIA in the 340-bp repeats (see Fig. 5), and R_1 (Table 1) in final concentrations of 0.1 μM. The PCR conditions were hot-start PCR (Ampliwax) touchdown initiation from 54 to 50°C annealing temperature (one cycle each) followed by 27 cycles of 45-s 94°C denaturation, 30-s 50°C annealing, and 1.5-min 72°C extension; and final extension for 10 min at 72°C. Ten microliters of this reaction mixture was ethanol precipitated and ligated into a T-A vector (pUC18, *Hin*dII). After electroporation, eight clones with an insert larger than 150 bp were selected and sequenced with the Tag Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Distribution of 340-bp repeat subfamilies in individual chromosome ends. Four chromosome ends (4R) were microdissected and DNA extracted as previously described. Before amplification the DNA was split into two parts that were treated with different combinations of restriction enzymes to generate monomers (see Results). Ethanol precipitates of such DNA were dissolved in 37 μl of water, of which 17 μl was used as a template for amplification. The conditions for the reaction were hot start and touchdown initiation from 59 to 56°C with a 1°C lowering of temperature each cycle and then 56°C for another 35 cycles (1-min 94°C denaturation, 30-s annealing, and 1-min 72°C extension) and final extension for 10 min at 72°C. The reaction was done in 100 μl, with two primers hybridizing all subfamilies at 0.2 μM: one was L3W, and the other was LI-IIA-C (Table 1). The reaction mixture also contained 2.5 mM $MgCl_2$, 200 μM dNTPs, 30 μCi of [^{32}P]dCTP, 0.5 U of Amplitaq, 0.001% gelatin, 63 mM KCl, and 12.5 mM Tris-Cl, pH 8.3.

RESULTS

Amplification of DNA from microdissected chromosome ends. If the <200-kb blocks of 340-bp tandem repeats reach the chromosome termini, it would be of considerable difficulty to clone the ends directly and to prove the origin of the cloned material. We therefore devised a procedure in which DNA extracted from microdissected chromosome ends was provided with an oligo(dC) tail which served to hybridize an R_0-R_1-$(dG)_{14}$ anchor. Primers corresponding to the anchored R_0 and R_1 sequences were then used for PCR together with primer hybridizing genomic DNA. Such amplification could be done in two directions, with either the genomic primer L3C or the complementary L3W, having homology to all subfamilies. The positions of the primer binding sites are shown for two subfamilies, M1 and D3, in Fig. 1. We assumed that tailing would occur at both ends of liberated DNA molecules. If, however, the blocks of repeats are intact, it is likely that tails on the centromere-proximal side of the repeat arrays are too far away from the repeats for amplification to be possible. Consequently, if PCR products are obtained preferentially with use of one of the genomic primers, this would provide a first indication that chromosome ends had been amplified.

Material was collected from each of the seven chromosome ends. The experiments were run together with a tailed plasmid control containing 10 D3 repeats in tandem (see Materials and Methods). Amplified DNA was separated in an agarose gel, blotted, and hybridized with the M1 sequence (which detects all subfamilies with roughly the same efficiency). Figure 2A shows that most of the amplified samples have a slowly migrating smear, but it also shows that some of them give a more distinct signal as well, with a maximum around 150 bp. Most of the smear was removed by nuclease S1 treatment, as seen in Fig. 2B, and is therefore likely to be due to single-strand extensions, expected to form from genomic primers with many hybridizing sites in long tandem arrays of repeats. For four chromosome ends (4R, 3R, 2R, and 2L) S1-resistant material was obtained only with the L3C primer, which hybridizes to the C-rich strand of the 340-bp repeat.

To relate signals from control plasmids to those generated by DNA from the chromosome ends, we had to estimate the number of genome equivalents in the extracted genomic DNA. This can be done (see the legend to Fig. 2) since the degree of polyteny has been measured in salivary glands from the closely related late-fourth-instar *Chironomus tentans* (9) and since the number of dissected pieces in each sample was known. On the basis of these calculations we estimate that chromosome amplifications were 10 to 25% as efficient as the plasmid controls. Such a difference could reflect the possibility that linearized plasmids with a short 3′ overhang are a better substrate for tailing than natural chromosome ends. The plasmid controls thus showed that PCR signals were not due to rare exceptional termini. They also made clear that L3W was a more efficient primer than L3C, which increases the significance of the difference in PCR signal in two directions. The predominance of DNA generated from chromosome ends with L3C, although a less efficient primer than L3W, could be due to amplification of chromosome termini, assuming that L3C is oriented towards the termini.

Effect of shearing DNA on amplification products. At least for those chromosome ends where amplification occurred with

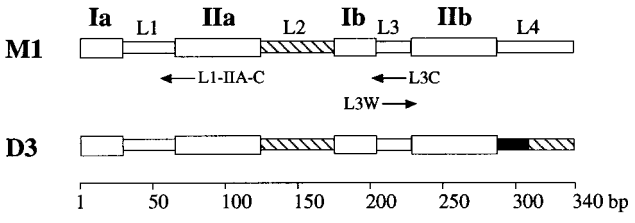


FIG. 1. Schematic comparison of the M1 and D3 subfamily units of the 340-bp repeat family. Ia, IIa, Ib, and IIb are subrepeats; the "a" units are similar to the corresponding "b" units; and L1 to L4 are linker regions, usually unique within the repeat unit. Hatched boxes are L2 sequences, which for D3 are in two positions, the common one and the subfamily-specific position, where L2 substitutes for L4 and where it contains a cluster of mutations, creating the subfamily-specific region ds3 in the beginning of L2 (filled box). Positions and orientation of primers used for amplification are shown.

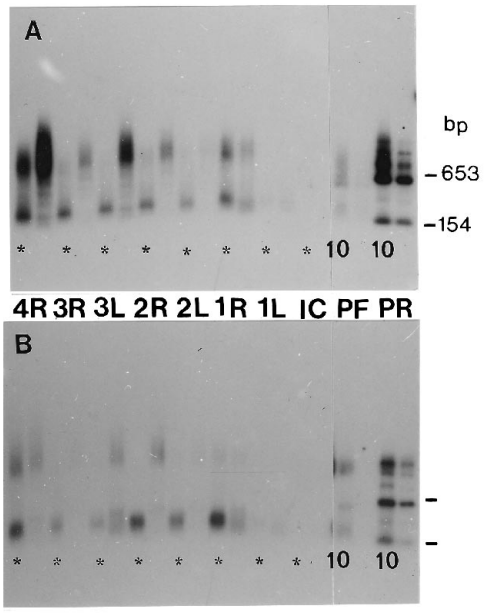


FIG. 2. Blots of electrophoretic separations of PCR amplifications of tailed DNA extracted from different chromosome ends of *C. pallidivittatus* salivary glands, hybridized with an M1 unit, the insert of pCp 306 (27). For each amplification 8 to 11 polytene chromosome ends were used. The primers for the amplifications were R_0 and L3C or L3W. Separations before (A) and after (B) S1 treatment of the PCR extracts are shown. Lanes 4R through 1L, chromosome 4, right telomere through chromosome 1, left telomere; lane IC, the two reactions for a control with internal chromosome pieces corresponding in amounts to the microdissected ends; lanes PF and PR, plasmid controls with L3C and L3W primers, respectively. Each plasmid reaction corresponds to 10,000 (labelled 10) or, to the right, 1,000 genome equivalents. The number of genome equivalents for each chromosome end was estimated to be 100,000 for 4R, 80,000 for 3L and 3R, and 60,000 for 2L, 2R, 1L, and 1R. (The basis for these estimations is that the largest size class in the closely related *C. tentans* has 16,000 chromatids per chromosome pair [9]. In the somewhat smaller *C. pallidivittatus*, this class is rare and most nuclei should have either 8,000 or 4,000 chromatids. If chromosomes belong to different replication steps, this can be seen in the microscope.) Amplifications with L3C, to the left in each pair of separations, are marked with asterisks; the right separations are with L3W.

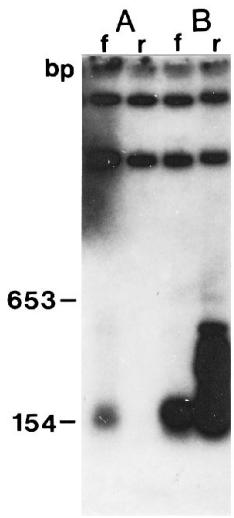


FIG. 3. Blots of electrophoretic separations of S1-treated PCR amplifications of DNA extracted from 10 chromosome 4R ends, carried out as described in the legend to Fig. 2. Tailings were done without (A) and with (B) prior vortexing for 30 s. Amplifications were with L3C (lanes f) and with L3W (lanes r). The high-molecular-weight bands represent plasmid DNA added to the amplification reactions for recovery control. The S1 treatment has been more efficient in these analyses than in Fig. 2B, as shown by the complete absence of the slowly migrating smear.

only one of the two primers, the results so far could be explained if DNA from chromosome termini had been amplified. If so, the interior of the repeat blocks was not available for tailing.

To investigate the correctness of this assumption, we had to devise a test to learn whether DNA throughout the whole block of repeats could become amplified in both directions if made available for tailing. For this purpose, we tailed 4R DNA before and after shearing and amplified the material in both directions with either L3C or L3W primer, as previously described for the experiments in Fig. 2B. Nonsheared DNA gave sequences hybridizing to an M1 repeat probe only with L3C. As expected, the amounts of material amplified with this primer increased considerably after shearing. Furthermore, products were obtained also with L3W after the treatment, in even larger amounts than with L3C (probably because of higher efficiency of L3W than L3C as a primer, as seen in previous experiments with a plasmid control). The results in Fig. 3 thus show that 4R DNA, if made available by shearing, can become tailed and amplified in both directions, also in interior parts of the repeat array. This in turn suggests that material generated by PCR from nonsheared DNA derives from chromosome termini, since it is obvious that the 340-bp arrays in such DNA have no internal breaks that can be tailed. The absence of amplification before shearing with the L3W primer also indicates that 340-bp repeats all have the same orientation within a repeat array.

Sequences of DNA amplified from chromosome end 4R. Further evidence that DNA generated with the L3C primer was terminal could be obtained by determining its subfamily identity. Direct cloning of the PCR products from 4R, one of the chromosome ends containing all four subfamilies (34), resulted in a low frequency of subfamily informative clones (the informative region lies in the initial part of L4 [Fig. 1]). This is because PCR amplification of repetitive sequences favors formation of shorter chains. To obtain a higher frequency of informative clones from 4R, we therefore reamplified the amplification product with primers R_1 and L1-IIA-C (Table 1), the latter lying closer to the downstream (right) end of L4 (Fig. 1). The results of sequencing eight clones, shown in Fig. 4, supported the view that the amplification products from 4R were derived from the chromosome terminus, since all sampled clones contained D3 DNA. They also indicated that termini are lying at different positions along the 340-bp repeat unit. The majority of clones obtained from the reamplified DNA hybridized to an M1 probe (26 of 50, the remainder being without visible inserts; 8 clones with the largest inserts were selected for sequencing). In all sequenced clones the tails were directly connected to D3 sequences. An absence of other DNA between the tails and the 340-bp repeats was also demonstrated in an investigation of 27 clones from the primary amplification. In all cases tails joined 340-bp repeat DNA directly (data not shown).

Subfamily composition of 340-bp repeats from chromosome end 4R. It was still possible that the recovery of only D3 units had an explanation other than their terminal localization, i.e., that the chromosome end 4R block of repeats contained predominantly D3 units and/or that such units were tailed or amplified more efficiently than other units. We therefore had to determine the subfamily composition in DNA generated by PCR from chromosome end 4R both before and after shearing and show how these data were related to the directly deter-

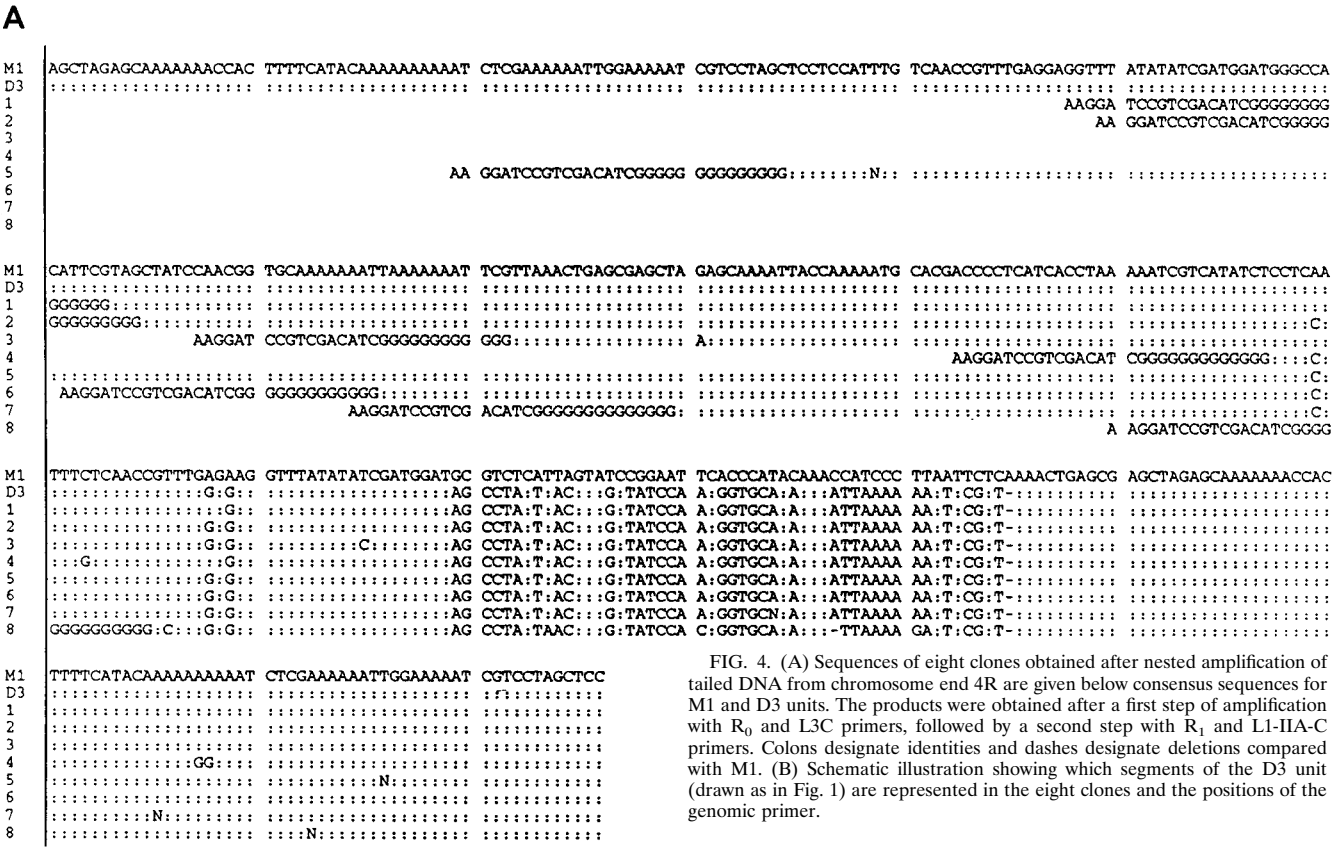
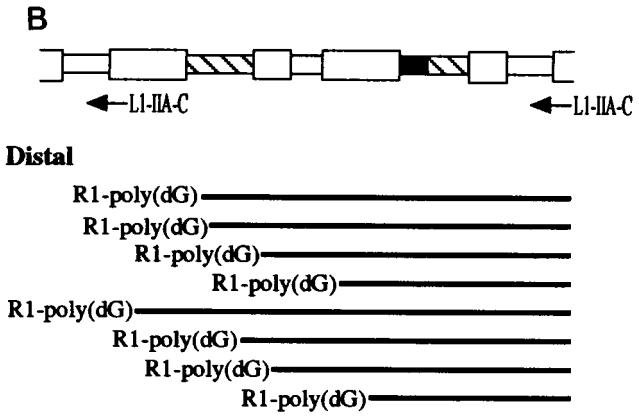


FIG. 4. (A) Sequences of eight clones obtained after nested amplification of tailed DNA from chromosome end 4R are given below consensus sequences for M1 and D3 units. The products were obtained after a first step of amplification with R_0 and L3C primers, followed by a second step with R_1 and L1-IIA-C primers. Colons designate identities and dashes designate deletions compared with M1. (B) Schematic illustration showing which segments of the D3 unit (drawn as in Fig. 1) are represented in the eight clones and the positions of the genomic primer.



mined subfamily composition for the whole array of 340-bp repeats. For this purpose we developed the following technique. We first converted the blocks of repeats in the extracted DNA to monomers with *Hae*III, which restricts the majority of repeats in all subfamilies (7, 27). The initial part of L4, containing subfamily-specific restriction sites (7, 34), lies in the middle of the released monomers. We used primers on both sides of this region for amplification. The products were then cut with the subfamily-specific restriction enzymes in different combinations, and the resulting fragments were separated by electrophoresis. This method, illustrated in more detail in Fig. 5, could be used either directly, with results shown in Fig. 6, or it could be applied to the products obtained after tailing and amplification of DNA before and after shearing, as shown below. When the subfamily composition was determined directly for the whole array of repeats with the aid of ^{32}P incorporation in the separated electrophoretic bands, we found M1 to account for 11% of all 340-bp repeats, D1 to account for 9%, D2 and D3 to account for 35% each, and the residual nonclassified fraction to account for 10%. This result can be compared to previously determined ratios between D1, D2,

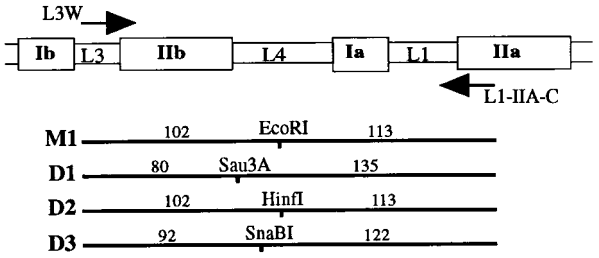
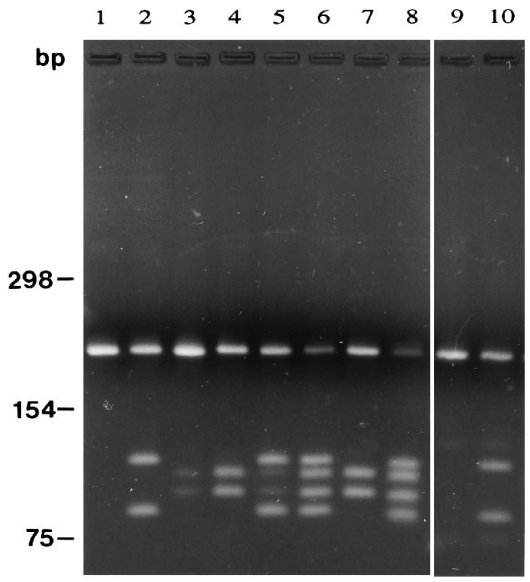


FIG. 5. Diagram of the procedure for determining subfamily composition in 340 bp of DNA from individual microdissected chromosome ends. Parts of two adjoining 340-bp repeat segments are shown on top, and amplification products representing the four main subfamilies, M1, D1, D2, and D3, are shown below. DNA is first extracted as described in Materials and Methods and then digested with *Hae*III (which cleaves the majority of repeats in all subfamilies in L2). The resulting monomers are then amplified with L3W and L1-IIA-C primer, which gives a 215-bp product. The amounts of repeats in the four subfamilies can be estimated by restriction enzyme digestion followed by gel electrophoresis. Sizes of fragments obtained by digesting the PCR products with different restriction enzymes are indicated in base pairs. All restriction sites are specific for the different families except *Eco*RI, which is present also in D1. If, however, the original PCR product is digested with *Sau*3A first, specifically recognizing ds1, D1 and some rare complex units (7) will not be amplified in the subsequent PCR.



Main, 215 bp band	M1 D2 D3	M1 D2 ---	--- D2 D3	M1 --- D3	--- D2 ---	M1 --- ---	--- --- D3	--- --- ---	M1 D2 D3	M1 D2 ---
Cleavage products	--- --- ---	--- --- D3	M1 --- ---	--- D2 ---	M1 --- D3	--- D2 D3	M1 D2 ---	M1 D2 D3	--- D1 ---	--- D1 D3

FIG. 6. Determination of subfamilies in the 340-bp repeat family of chromosome end 4R from the animal used for the experiments in Fig. 3. Half of the DNA extracted from four chromosome 4R ends was restricted with *Hae*III and *Sau*3A (lanes 1 to 8), amplified as described in Materials and Methods, and run in a 3.2% NuSieve GTG agarose gel without further treatment (lane 1) and after restriction with *Sna*BI (lane 2), *Eco*RI (lane 3), *Hin*fI (lane 4), *Sna*BI plus *Eco*RI (lane 5), *Sna*BI and *Hin*fI (lane 6), *Eco*RI plus *Hin*fI (lane 7), and *Sna*BI, *Eco*RI, and *Hin*fI (lane 8). Lanes 9 and 10, amplifications after *Hae*III restriction of the other half of the extracted DNA, where instead *Sau*3A (lane 9) and *Sau*3A plus *Sna*BI (lane 10) were added after amplification. The amounts of DNA in the upper and the lower bands were determined by ^{32}P incorporation with [α-^{32}P]dCTP precursor. The present procedure results in a distribution of subfamilies as shown in the table below the ethidium bromide-stained gel.

and D3 of 1:3:3 in total DNA (8). We can consequently exclude the possibility that the predominance of D3 clones was due to biased subfamily composition in chromosome end 4R, which strengthens our conclusions that they derive from the chromosome terminus.

Subfamily composition in PCR-generated 4R DNA before and after shearing. Subfamily compositions were then determined in DNA generated by PCR from the experiment in Fig. 3 before and after shearing. Figure 7 shows that essentially only D3 DNA was obtained before treatment, whereas DNA representing different repeat subfamilies was generated from sheared DNA, roughly in proportions characteristic for the intact chromosome 4R ends from the same animal. Consequently we were able to confirm previous conclusions that 340-bp repeat DNA extends to the 4R terminus in *C. pallidivittatus* and eliminate alternative interpretations.

The termini are to the left of the 340-bp units shown in Fig. 1 and 5, where the C-rich strand is diagrammed going from left (5′) to right (3′). This means that the G-rich strand is oriented with the 3′ end towards the chromosome terminus.

DISCUSSION

We have obtained evidence that the right end of salivary gland chromosome four from *C. pallidivittatus* contains 340-bp tandem repeats at its extreme terminus. Only the oligonucleotide hybridizing the C strand, not the complementary one, amplified DNA towards an added tail. After shearing of the genomic DNA, PCR was, however, productive in both directions. This strongly indicates that the array of 340-bp repeats was intact before shearing, excluding the possibility that internal breaks were tailed. In agreement with this, only the most peripheral subfamily of repeats was seen in amplified material, although all subfamilies have about equal chances to amplify, as shown by the shearing experiments. In all cloned material, 340-bp DNA was directly connected to the added tails at different positions along the whole repeat.

The same repeat family is present at the cytological level at all nontelocentric chromosome ends (27), and it is likely therefore that all such ends terminate with 340-bp repeats.

Repetitive sequences may be eliminated during polytenization in members of the genus *Drosophila*. In chironomids, however, such DNA has been found to replicate to the same extent as bulk DNA (13, 28, 32). For a sibling species to *C. pallidivittatus*, *C. tentans*, for which diploid tissue-cultured cells are available, similar amounts of DNA hybridizing to the 340-bp repeat were found in DNA from larvae (mainly polytene cells) and the cultured cells (27). Consequently, polytene cells are likely to be representative for diploid cells. It might nevertheless be argued that a hypothetical telomeric DNA of a different kind, present in the germ line, was lost in somatic chromosomes. Somatic cells may shorten their telomeres as a

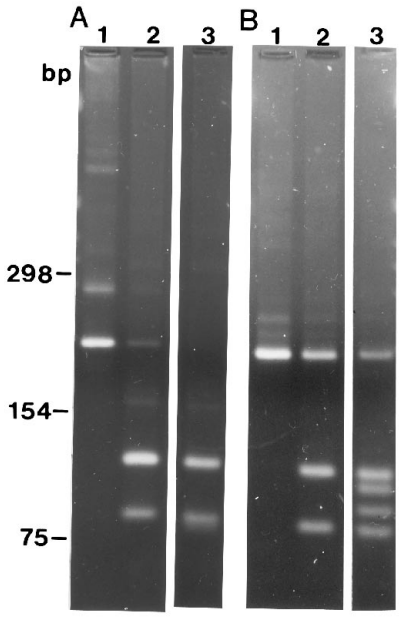


FIG. 7. Subfamily composition in material amplified from DNA tailed before (A) and after (B) vortexing for 30 s. The products obtained from the reactions primed with L3C and R_0 shown in Fig. 3 were used to analyze subfamily composition as in Fig. 5, i.e., they were first restricted with *Hae*III and *Sau*3A and then reamplified with primers L3W and LI-IIA-C. Lanes 1, products without further treatment; lanes 2, separations after digestion with *Sna*BI, which cuts D3 units. In panel A, lane 2, most of the 215-bp PCR product has been converted to bands of the size characteristic for D3 (122 and 92 bp), whereas in panel B, lane 2, much undigested material remains. Lanes 3, the same DNA after digestion with *Eco*RI, *Hin*fI, and *Sna*BI, which cleave 340-bp repeat DNA in M1, D2, and D3, respectively. In panel A, 3, this enzyme combination results in approximately the same pattern as for panel A, lane 2, whereas for sheared DNA, in panel B, lane 3, the 113- and 102-bp bands characteristic for M1 and D2 appear in addition to nondigested 215-bp non-D3 DNA. The electrophoretic patterns are not as clean as in Fig. 6, probably because the amplified DNA was created by PCR of PCR products, i.e., double rounds of amplification.

function of replicative age. However, this does not normally lead to complete elimination of telomeric DNA (see references in reference 12).

Our results suggesting that 340-bp repeats terminate chromosomes are of interest in relation to their assumed evolutionary origin. These units may have evolved from short telomeric repeats (22). In the present repeats it is still possible to distinguish between a G-rich and a C-rich strand, and it might be significant that the orientation of these strands is the same as in typical telomeric DNA. The orientation agrees with DNA flanking the tandem arrays being centromere proximal to the 340-bp repeats (22).

It has been suggested that short G-rich repeats are important for telomere function by providing special secondary structures (see references in reference 4). Results from another dipteran, *D. melanogaster*, show that such a hypothetical function cannot be universal, a conclusion which is supported by functional analysis of *Kluyveromyces lactis* mutants (18). These conclusions are reemphasized by our results.

Interestingly, 340-bp repeats are detectable at only seven of the eight chromosome ends, the remaining, telocentric 4L end containing 155-bp tandem repeats, present also in other centromeres (26). Although it is not yet known whether the 155-bp repeat is terminal, it is likely that different kinds of DNA are used as chromosome terminal DNA in one and the same organism like in *D. melanogaster*, where two different retrotransposons, HeT-A and TART, have been found at the chromosome ends (3, 30).

Complex repeats of the present type are unlikely to be generated by telomerase, the longest unit known to be formed in this way being 25 bp in the yeast *K. lactis* (18). As suggested for *T. thermophila* mitochondrial DNA (19, 20), unequal recombination might restore longer repeats. If this applies to *C. pallidivittatus* telomeres, it may be necessary to assume preferential loss of genotypes with small amounts of 340-bp repeats at some telomere. This would imply a certain genetic load that could nevertheless be relatively small. This is because there are large variations in DNA amounts between and within telomeres, in the 100-kb range (8). The replication-linked DNA loss, which per fly generation is of the order of 50 to 100 bp in *D. melanogaster* (1, 14), is small in relation to these fluctuations. Another possibility is that gene conversion generates 340-bp repeats. The mosaic structure of complex 340-bp repeats and the efficient sequence homogenizations within and between subfamilies suggest that such processes operate in *C. pallidivittatus* telomeres (7). In contrast to DNA regeneration by unequal crossing over, gene conversion is not necessarily accompanied by a genetic load. A third possibility, however, that chironomid telomeres are extended by reverse transcriptase-related mechanisms, as for other telomeres for which the main restoration mechanism has been established, cannot be excluded.

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